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RESEARCH ARTICLE

Evaluation of the Antioxidant and Antimicrobial Activity of *Thunbergia fragrans* Roxb Leaves Extract

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Abstract

Nowadays plants are the traditional product that has little or no side effects in modern medicine. The *Thunbergia fragrans* belong to the family *Acanthaceae* which contains more biologically active phytoconstituents. The plant leaves were extracted with different solvents including ethanol, aqueous, hexane and ethyl acetate. These extracts have been subjected to phytochemical study. The results of the qualitative phytochemical analysis indicate that the ethanol extracts provided more phytoconstituents. The phytochemical quantitative analysis showed that the ethanol had a higher amount of flavonoid. The ethanol extract of *Thunbergia fragrans* contained antioxidant and antibacterial activity. The GCMS study for the compound identification was studied for all four extracts. The result revealed the presence of various biologically active compounds in the ethanol extract.

Keywords: Antioxidant, Antimicrobial, Ethanolic Extract, *GCMS, Thunbergia fragrans*

1. Introduction

Plants are an important resource for traditional medicine which contains various medicinal properties[1]. *Thunbergia fragrans* is a fast-growing herbaceous vine which is widely grown as an ornamental plant in tropical and subtropical regions of the globe but is widespread in moist disturbed areas as well. Born in India and Ceylon, it is widely cultivated for its attractive white flowers in the tropical and subtropic regions. It belongs to the Acanthaceae family and has some biological function, including antidiabetic, antipyretic, antidote and anti-inflammatory activities. Along with these activities the *T. fragrans* have promising anticancer activity because of the higher flavonoid content in the plant[2,3]. This study was to investigate the chemical constituents of *T. fragrans* leaves extracted with different solvents such as aqueous, ethanol, hexane and ethyl acetate. Standard procedures were used for the qualitative and quantitative determination of chemical constituents. The purpose of the present study was to identify and characterize the phytoconstituents of various *T. fragrans* extracts[4–8]. The qualitative and quantitative phytochemical analysis and the GCMS analysis was performed to identify the compounds present in the extracts[9]. Finally, the antioxidant and antibacterial activity of *T. fragrans* extract was evaluated.

2. Materials and Methods

2.1 Plant Collection

The disease-free healthy leaves of *Thunbergia fragrans* were collected from the botanical garden in Thanjavur, Tamil Nadu, India.

2.2 Chemicals and Glassware

All the analytical grade chemicals and the glasswares were purchased from Nice chemicals and sterilized before use.

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2.3 Plant Processing

Fresh leaves of *Thunbergia fragrans* were washed thoroughly with distilled water. The leaves were dried in a hot air oven at 45 ºC. These dried leaves were powdered using a mechanical grinder. Then the powder material was extracted in aqueous, ethanol, hexane and ethyl acetate using Soxhlet extractor. The dried extract was stored in an airtight container and maintained at 4 ºC in a refrigerator for further use. The standard methods such as qualitative and quantitative analysis was used to analyze the extract[10–12].

2.4 Qualitative Analysis of Chemical Constituents of *Thunbergia fragrans* Leaves Extracts

2.4.1 Test for Alkaloids

Plant extract (0.2 g) was warmed and filtered into a test tube containing 2 % sulfuric acid (3 mL). Few drops of Dragon dwarf reagent. The appearance of orange-red precipitates confirmed the presence of alkaloids[13,14].

2.4.2 Test for Terpenoids

2 mL of chloroform was added to 0.5 g of the extract in a test tube. The walls of test tube will form a layer when the concentrated sulfuric acid is added. The appearance of a reddish-brown coloured confirmed the presence of terpenoid[13,14].

2.4.3 Test for phenol

To a 5 ml of distilled water, 0.5 ml of plant extracts was added. The mixture was boiled for 10 min and filtered. A few drops of ferric chloride solution were added to 2 ml of filtrate. A greenish blue or violet color appearance indicated the presence of phenolic hydroxyl group[13,14].

2.4.4 Test for Tannins

In a test tube, small quantities of the plant extracts are added into 3 mL of water and placed on a boiling water bath for 5 min. The mixture was filtered and ferric chloride was added. A dark green colour indicated the presence of tannins[13,14].

2.4.5 Test for Quinones

The quinone test was carried out by the addition of 1 mL extract to 1 mL sulfuric acid. Red colour formation revealed the presence of quinines[13,14].

2.4.6 Test for Saponins

In a test tube, approximately 0.2 g of the plant extract and 5 mL of distilled water was added. The above mixture was shaken well and then filtered. The filtrate was boiled. The frothing indicated the presence of saponins[13,14].

2.4.7 Test for Reducing Sugars

2 mL of crude plant extract was added to 5 mL distilled water, shaken well and filtered. The filtrate was boiled for two minutes with 3–4 drops of Fehling solutions, A and B. The formation of orange-red precipitate confirmed the presence of reducing sugars[13,14].

2.4.8 Test for Anthraquinone

To 10 ml of benzene, 0.5 g crude powder and 0.5 ml of 10 % of ammonia was added and filtered. Filtrate were well shaken and the formation of violet colour in the layer phase indicated the presence of anthraquinones[13,14].

2.4.9 Test for Steroids

To 0.5 g of ethanolic extract (plant), 2 mL of acetic anhydride and 2 mL of sulfuric acid was added and mixed well. Violet to blue-green colour formation indicated the presence of steroids[13,14].

2.4.10 Test for Glycoside

A few mL of plant extract was added to water, glacial acetic acid, ferric chloride and concentrated sulphuric acid. The formation of the brown ring at the bottom suggested the presence of cardiac glycosides[13,14].

2.4.11 Test for Flavonoids

A 0.2 g of plant extract and was dissolved in cold diluted sodium hydroxide solution in a test tube. Diluted hydrogen chloride was added to the above solution. The presence of flavonoids was confirmed by the appearance of a colourless yellow solution[13,14].

2.4.12 Test for Protein

A few drops of 4 % sodium hydroxide and 1 % copper sulphate solution were added to 2–3 mL of plant extracts. Violet or pink appearance suggested the presence of proteins[13,14].

2.5 Chemical Constituents of *Thunbergia fragrans* Leaves Extracts

2.5.1 Estimation of Total Alkaloids

Dimethyl sulfoxide and 1 mL of hydrochloric acid (2N) were used to dissolve 1 mg/mL equivalent of the plant sample. The mixture was filtered through the filter medium and transferred to separating funnel. A 5 ml bromo-cresol green solution and a 5 ml of phosphate buffer was added to the filtrate with 1, 2, 3 and 4 ml chloroform. The mixture was vigorously shaking and collected in a 10 ml volumetric bottle and diluted to the $CHCl_3$ amount. At 470 nm with a UV-Vis spectrophotometer, the absorbance for the test and regular solutions is calculated. A series of benchmark atropine solutions were developed as stated previously (20, 40, 60, 80 and 100 µg/ml)[13].

2.5.2 Estimation of Total Flavonoids

The aluminium chloride colorimetric assay measured the total flavonoids. The reaction combination is 1 ml extract and 4 ml of distilled water was subjected to a 5 % sodium nitrite solution of 0.3 ml. 0.3 ml of 10% aluminium chloride is mixed after five minutes. Treatment and dilution of 2 ml of 1 M sodium hydroxide to 10 ml of distilled water were carried out after 5 minutes. At 510 nm with the UV-Vis spectrophotometer, the absorption of test solutions and regular solutions was calculated against the reagent blank. A benchmark quercetin solution package was developed as stated above (20, 40, 60, 80 and 100 µg/ml)[15].

2.5.3 Estimation of Total Saponins

In a conical flask containing 100 ml of 20 % aqueous ethanol, add 20 g of powdered sample. The solution was heated at 55° C for 4 hours. The solution was then filtered and extracted with 20 % ethanol of 200 ml. The two extracts were then combined and the solvent evaporated up to 40 ml of extract volume. Further removing the condensed filtrate with 20 ml diethyl retrieved the aqueous layer, while the ether was discarded. By adding 60 ml of n-butanol, the aqueous extracts were purified. It was then washed with 5% aqueous sodium chloride, twice 10 ml[16].

2.5.4 Estimation of Total Tannins

Folin-Ciocalteu method determined the overall tannins. Around 0.1 ml of this sample had a distilled water content of 7.5 ml and Folin-Ciocalteuphenol (0.5 ml) and a 35 per cent Na_2CO_3 solution of 1 ml and diluted with 10 ml distilled water. The mixture was well shaken and stored for 30 minutes at room temperature. At 725 Nm with UV-Vis spectrophotometer, absorption for testing and standardised solutions was measured against the blank. Gallic acid (20, 40, 60, 80, and 100 µg/ml) was formulated in the same way as the previously mentioned reference standard solutions were developed[17].

2.5.5 Estimation of Total Phenolics

Folin-Ciocalteu assays were used to assess the total phenolics. Approximately 1 ml extract has been added, 1ml Folin-Ciocalteu phenol reagent was shaken with 9 ml distilled water. After 5 minutes, the mixture was treated with 10 ml of 7 per cent of Na_2CO_3 and up to 25 ml in volume. The reagent blank at 550 nm with a UV-Vis spectrophotometer was incubated for 90 min at room temperature, the absorption for testing and regular solution. Some standard Gallic Acid solutions have been developed in the same way as before (20, 40, 40, 60, 80 and 100 µg/ml)[18].

2.6 GC-MS Analysis

Gas chromatography and mass spectrometry QP2010 Plus, Shimadzu, Japan fitted with the RTX–5 MS GC capillary column (5.1 % diphenyl/ 95.0 % dimethyl polysiloxane) of 0.5 µm in diameter and 30 m in length have been evaluated for identifying and quantification of *Thunbergia fragran's* chemical components. Working conditions: the temperature was kept between 40–290° C and progressively increased to 8° C/min. The oven column and the injection were set at 100° C and 270° C. Helium is used as carrier gas (mobile phase) at a rate of 1 ml/min. Injection mode has been set to a fraction

of 20. Working conditions for MS: the temperature of ion sources and interfaces was set to 200°C and 260° C. Cutting time for solvents was set to 4 min and the voltage for detectors was set at 0.1 kV. Conditions for injection: injection 1 µL, injection 10 µL, injecting temperature 240° C; weight range 20–300 m/z[19–21].

2.7 Antioxidant Activity

The radical scavenging activity of *Thunbergia fragrans* extract was calculated by DPPH assay. The stock solution of plant extract was prepared at various concentration. Adding 1 ml of DPPH in each concentration of the extract was incubated at 30 minutes in a dark room. Finally, the absorbance of the plant extract was calculated at 520 nm. Ascorbic acid was used as a standard solution[22]. The scavenging activity percentage was calculated using the formula.

$$\text{Percentage inhibition} = \frac{(\text{Absorbance of control} - \text{Absorbance of sample}) \times 100}{\text{Absorbance of control}}$$

2.8 Antibacterial Activity

2.8.1 Disk Diffusion Method

The antibacterial activity of *Thunbergia fragrans* ethanolic leaf extract was studied against two bacterial strains *Escherichia coli* and *Staphyloccocus aureus*. The antibacterial assay was carried out by disk diffusion method as per the study performed by Chan E.,[23]. Finally the zone of inhibition was calculated.

3. Result and Discussion

3.1 Qualitative Determination

Table 1 represents the presence of chemical constituents of *T. fragrans* leaf extract of various solvent (Aqueous, ethanol, hexane, ethyl acetate). The result revealed the presence of various phytoconstituents like alkaloid, flavonoid, cardiac glycoside, saponin, tannin and phenolics. Moreover, ethanol extract showed a high number of secondary metabolites compare with other extracts. Alkaloids, flavonoids and amino acid were present in all the extracts.

3.2 Quantitative Determination

The quantitative phytochemical analysis was performed to various leaves extracts of *T. fragrans* were presented in Table 2. Quantitative evaluation indicates the presence of Alkaloids, flavonoids, saponins, tannins and phenols. Flavonoids and phenols were highly present in the ethanol extract but the tannin content was low. Comparatively ethanol extract showed more number of chemical constituents among all the extract. Further these extracts were taken to the GCMS quantification.

Table 1. Preliminary Phytochemical screening for the various extract of *Thunbergia fragrans* leaves.

S. no	Chemical constituent	Aqueous extract	Ethanol extract	Hexane extract	Ethyl acetate extract
1	Alkaloids	+	++	+	+
2	Flavonoids	+	++	+	+
3	Terpenoids	-	+	-	+
4	Phenol	+	++	++	-
5	Tannins	+	+	-	-
6	Quinones	+	-	+	+
7	Saponins	-	+	-	-
8	Reducing sugar	+	+	-	-
9	Anthraquinones	-	+	-	+
10	Steroids	-	-	+	+
11	Glycoside	-	+	-	-
12	Protein and Free amino acid	+	++	+	+

Note: (++) denotes highly present; (+) denotes present; (-) denotes not detected.

Table 2. Quantitative determination of chemical constituents of *Thunbergia fragrans* Leaf extracts

Extracts	Alkaloids	Flavonoid	Saponins	Tannins	Phenols
Aqueous	2.5±0.25	4.98±0.5	5.40±0.15	0.7±0.6	18.25±0.35
Ethanol	6.25±0.15	22.5±0.85	3.0±0.25	2.5±0.35	15.35±1.5
Hexane	0.7±0.2	10.58±1.8	6.40±0.25	0.8±0.65	7.40±0.65
Ethyl acetate	1.05±0.65	8.40±0.6	2.0±0.15	3.0±0.45	10.40±0.6

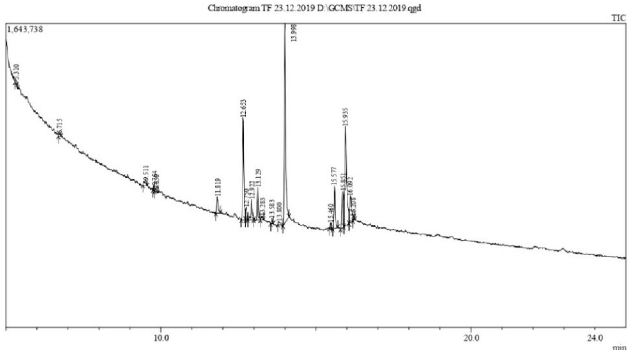


Figure 1. GCMS chromatogram of Ethanolic extract of *Thunbergia fragrans* leaves.

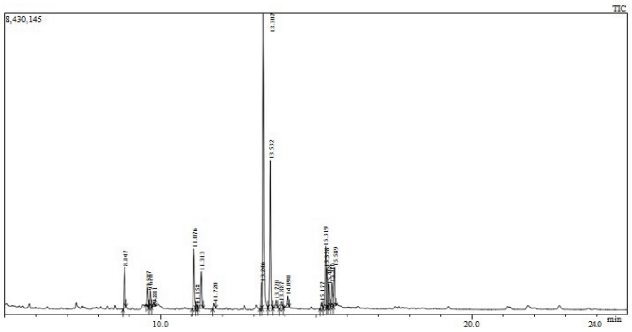


Figure 2. GCMS chromatogram of aqueous extract of *Thunbergia fragrans* leaves.

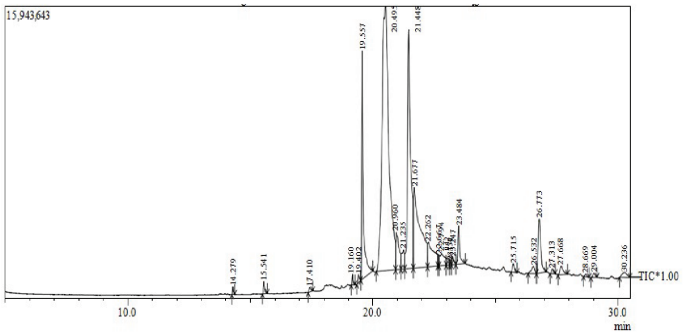


Figure 3. GCMS chromatogram of ethyl acetate extract of *Thunbergia fragrans* leaves.

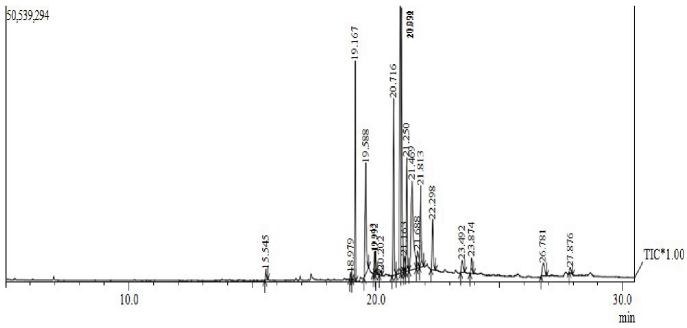


Figure 4. GCMS chromatogram of hexane extract of *Thunbergia fragrans* leaves.

Table 3. Quantitative determination of chemical constituents of *Thunbergia fragrans* Leaf Ethanol extract by Gas chromatography Mass spectrometry

Peak	Retention time	Area %	Name
1	5.310	0.74	3-(3-OXO-3H-BENZO[F]CHROMEN-2-YL)
2	6.715	0.40	[1R*,2R*]-1-acetyl-1,2-dihydroxycyclohex-3-
3	9.511	1.14	4,5-Heptadien-2-0l, 3,3,6-tnmethyl- (CAS)
4	9.764	0.41	Borane, triethyl- (CAS) Triethyl borane
5	9.850	0.47	ARGON, MOL (AR2)
6	11.819	2.56	Hexadecanoic acid (CAS) Palmitic acid
7	12.653	13.42	NEOPHYTADIENE
8	12.750	1.83	2-Heptadecanone (CAS) 2-HEPTADECANO
9	12.922	3.38	2-Hexadecen-1-ol,3,7,11,15-tetramathyl-, [R-
10	13.129	4.22	2-Hexadecen-1-ol,3,7,11,15-tetramethyl-, [R-
11	13.283	0.70	1,9-Nonanediol (CAS) N-NONANE-1,9-DIO
12	13.583	0.66	Methyl 11-(2,3-Dideuterocyclopen

Table 3. (Cont)

Peak	Retention time	Area %	Name
13	13.800	0.47	Bicyclo[4.1.0]heptane, 7-butyl-(CAS) Norcar
14	13.998	28.98	Hexadecanoic acid (CAS) Palmitic acid
15	15.460	0.55	9,12,15-Octadecatrienoic acid, methyl ester (c
16	15.577	5.88	2-Hexadecen-1-OL, 3,7,11,15-Tetrame
17	15.851	6.69	11,14-Eicosadienoic acid, methyl ester (CAS)
18	15.935	21.35	9,12,15-Octadecatrienoic acid, methyl ester (Z)
19	16.092	5.61	Docosanoic acid (CAS) Behenic acid
20	16.208	0.53	7-Ethyl-3-Methyl-2-Nitro-4-Oxo-4,7

Table 4. Quantitative determination of chemical constituents of *Thunbergia fragrans* Leaf Aqueous extract by Gas chromatography Mass spectrometry

Peak	Retention time	Area %	Name
1	8.847	4.18	Phenol, 3,5-bis(1,1-dimethylethyl)- (CAS) 3,5
2	9.587	2.10	1,2-Benzoldicarbonsaeure, Di-(He
3	9.676	1.91	1,2Benzoldicarbonsaeure, Di-(He
4	9.801	0.51	Hexane, 2,4,4-trimethyl- (CAS) 2,4,4-Trimeth
5	11.076	6.98	1-Tetradecanamine, N,N-dimethyl- (CAS) DI-
6	11.158	0.56	Oxaloacetic acid
7	11.313	4.39	Pentanoicacid, 4-methyl-, methyl ester (CAS)
8	11.720	0.71	Hexadecanoic acid (CAS) Palmitic acid
9	13.246	2.75	7,10-Hexadecadienoic acid, methyl ester(CAS)
10	13.307	33.65	Palmitic acid
11	13.532	15.69	Cis aconitic acid
12	13.720	1.12	(3-Tert-Butyl-5-Hydroxymethyl-C
13	13.887	1.13	1,3-Propanediol, 2-ethyl-2-(hydroxymethyl)-
14	14.090	1.60	1,2-Benzenedicarboxylic acid, dibutylester(C
15	15.177	0.82	9-Octadecene, (E)- (CAS)
16	15.319	7.66	Propane, 1,1,3-triethoxy-
17	15.358	3.92	17-Octadecenoic acid, methyl ester (CAS)ME
18	15.408	2.60	9,12,15-Octadecatrienoic acid, methyl ester
19	15.516	3.09	2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-, [R-
20	15.589	4.64	Methyl Stearate

Table 5. Quantitative determination of chemical constituents of *Thunbergia fragrans* Leaf Ethyl acetate extract by Gas chromatography Mass spectrometry

Peak	Retention time	Area %	Name
1	14.279	0.15	2,6-Difluorobenzoic acid, tridec-2-ynyl ester
2	15.541	0.35	1,2-Benzenedicarboxylic Acid, Die
3	17.410	0.19	Tetradecanoic acid
4	19.160	0.23	Hexadecanoic acid, methyl ester
5	19.402	0.26	cis-9-Hexadecenoic acid
6	19.557	8.20	n-Hexadecanoic acid
7	20.495	42.95	Butyl 9,12-octadecadienoate
8	20.960	2.67	9,12-Octadecadienoic acid (Z,Z)-, methyl este
9	21.235	1.22	Octadecanoic Acid, Methylester

Table 5. (Cont)

Peak	Retention time	Area %	Name
10	21.448	17.52	cis-9-Hexadecenal
11	21.677	12.56	Octadecanoic acid
12	22.262	3.33	Oxirane, [(dodecyloxy)methyl]-
13	22.667	0.32	Eicosanoic acid, 2-hydroxyethyl ester
14	22.794	1.32	Carvacrol
15	23.042	0.38	2-(Dimethylamino)Ethyl 1-Adama
16	23.150	0.18	4,7,10,13,16,19-Docosahexaenoic acid, methy
17	23.247	0.35	3-Cyclopentylpropionic acid, 2-dimethylamin
18	23.484	1.59	Hexadecanoic Acid, 2-Hydroxy-1,3
19	25.715	0.37	2,2-Dimethylpropanoic acid, heptadecyl ester
20	26.532	0.53	2,6,10,14,18-Pentamethyl-2,6,10,14,18-

Table 6. Quantitative determination of chemical constituents of *Thunbergia fragrans* Leaf Hexane extract by Gas chromatography Mass spectrometry.

Peak	Retention time	Area %	Name
1	15.545	0.63	1,2-Benzenedicarboxylic Acid, Die
2	18.979	0.35	9-Hexadecenoic Acid, Methyl Est
3	19.167	8.71	Hexadecanoic Acid, Methylester
4	19.588	7.89	n-Hexadecanoic acid
5	19.943	1.16	Glycidol stearate
6	19.992	1.06	cis-10-Heptadecenoic acid, methyl ester
7	20.202	0.36	Heptadecanoic acid, methyl ester
8	20.716	9.90	Methyl 2-octylcyclopropene-1-heptanoate
9	20.994	20.79	Campesterol
10	21.032	14.24	9-Octadecenoic Acid, Methyleste
11	21.163	1.13	9,12-Octadecadienoic acid (Z,Z)-
12	21.250	6.53	Pentadecanoic acid, 14-methyl-, methyl ester
13	21.469	12.11	cis-13,16-Docasadienoic acid
14	21.688	1.79	Octadecanoic acid
15	21.813	5.38	Ethyl 2-dibromomethyl-6-cyano-7-ethoxy-5 phenyl-1,8-n aphthyridine-3-carboxylate
16	22.298	3.44	cis-10-Nonadecenoic acid, methyl ester
17	23.492	1.14	Ethyl 9,12,15-octadecatrienoate
18	23.874	1.13	Eicosanoic Acid, Methyl Ester
19	26.781	1.80	(R)-(-)-14-Methyl-8-hexadecyn-1-ol
20	27.876	0.47	Docosanoic Acid, Methyl Ester

The above-mentioned values are in percentage by conversion of mg/standardgram equivalent.

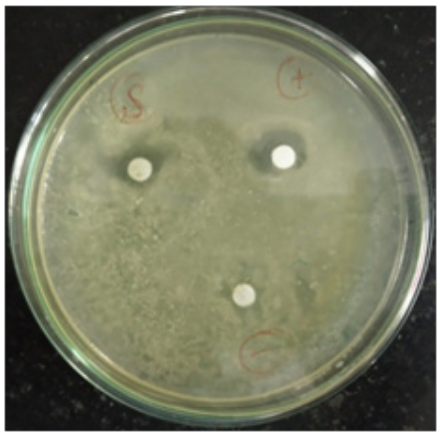
Figures 1–4 and Tables 3–6 represents the chromatogram and percentage composition of chemical constituents of ethanol, Aqueous, Ethyl acetate and Hexane leaf extract of *Thunbergia fragrans* respectively. The chromatogram peak compared with the Wiley and NIST database of GCMS Library (spectrum installed). Twenty major compounds were identified and characterized by GCMS. The identified compounds are listed in Tables 3-6 in ascending retention time order. The major compounds were present in the ethanol extract were Neophytadine, Hexadecanoic acid and 9,12,15-Octadecatrienoic acid.

The qualitative and quantitative results revealed ethanol is the most suitable solvent to extract the

Table 7. Antioxidant activity of ethanolic extract of *Thunbergia fragrans* leaf.

Concentration (µg/ml)	Ascorbic acid	Scavenging activity (µg/g)
20	220.35 ± 3.1	190.15±1.5
40	225.65 ± 2.9	206.30±0.5
60	258.55 ± 3.5	229.40±2.5
80	346.85 ± 2.5	260.80±1.5
100	344.55 ± 1.6	275.50±0.5

Values are mean ± S.E.M, n = 3



Figures 5 and 6. Antibacterial activity of *Thunbergia fragrans* ethanolic extract against *Staphyloccocus aureus* and *Escherichia coli*

Table 8. Antibacterial activity of *Thunbergia fragrans* ethanolic leaf extract

Organism Name	Standard (mm)	Sample (Extract) (mm)	Control (mm)
Staphylococcus aureus	11	18	-
Escherichia coli	11	15	-

chemical constituents of *Thunbergia fragrans* leaves. Therefore the ethanol extract is subjected to the DPPH assay for the identification of free radical scavenging activity. Table 7 represents the scavenging activity of *Thunbergia fragrans* at a different concentration such as 20, 40, 60, 80, 100. All concentration had scavenging activity moreover 100(µg/ml) concentration had more scavenging activity (275.50±0.5) when compared with others.

3.3 Antibacterial Activity

The disk size was 8 mm and streptomycin used as a standard. The antibacterial activity of ethanol extract of *Thunbergia fragrans* leaves was found significant against two bacteria, *Escherichia coli, Staphyloccocus aureus* (Table 8, Figures 5, 6). The extract showed very high activity in *S. aureus,* with the zone of inhibition of 18 mm diameter in 100 µg concentration. The standard (10 µg streptomycin) showed zone of inhibition 11 mm in both the *sp.* respectively. Extract showed prominent zone of inhibition.

4. Conclusion

Thunbergia fragrans extracts were quantified for the identification of medicinally important phytochemicals. *T. fragrance* leaves were evaluated by the qualitative and quantitative method. GCMS

results revealed the presence of chemical constituents with the exact composition of different solvents such as ethanol, aqueous, hexane and ethyl acetate. Tables 1 and 2 results revealed ethanol is the most suitable solvent to extract the chemical constituents of *T. fragrance* leaves. Moreover, quantitative result of the ethanolic extract showed a higher amount of flavonoid which had a promising anticancer activity. Tables 7 and 8 revealed *Thunbergia fragrans* ethanol extract had strong antioxidant and antibacterial activity. Further investigation would explore the anticancer activity of *T. fragrance* leaves through *in silico* and *in vitro* studies.

5. Acknowledgement

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